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Upregulation of Autophagy by Angiotensin II Triggers Phenotypic Switching of Aortic Vascular Smooth Muscle Cells

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Abstract

Background: Angiotension II (AngII) has important roles on Vascular Smooth Muscle Cell VSMC) biological functions. It has been indicated to promote the synthetic phenotype of VSMC, but the underlying mechanism is still needed to be elucidated.

Methods and Results: In this study, we found AngII infusion promotes a increased form of autophagy characterized by increased expression of Beclin1 and LC3-II in the cultured human aortic vascular smooth muscle cells. The growth of autophagy stimulated by AngII could enhance implication of synthetic proteins in VSMCs, while inhibition of autophagy with spautin-1or 3-MA can encourage the stability of the contractile phenotype in AngII-treated VSMCs.

Conclusion: These results suggest that up regulation of autophagy by AngII contributes to the increase of synthetic phenotype, which triggered the functional change in VSMCs.

Keywords: Angiotension II; Autophagy; VSMC phenotype switch; Beclin1; LC3

Introduction

Vascular Smooth Muscle Cells (VSMCs) phenotype switching has been demonstrated to be the major contributor of many cardiovascular diseases including atherosclerosis, aneurysm and aortic dissection [1,2]. Changing of VSMCs from a contractile phenotype to a synthetic phenotype triggers their migration to the intima, promotes their proliferation and induce synthesis of extracellular matrix protein, which ultimately results in impaired contractility of vascular function [1]. Although considerable factors taking part in the phenotype transitions of VSMCs, angiotensin II (AngII) has played profound role in phenotype modulation of VSMCs, which encourages the phenotype switching of VSMCs from contractile type to synthetic type and inhibition of AngII signaling is major player in VSMC proliferation and migration [3-5]. Hence, understanding how AngII manipulate VSMC phenotype is very essential for developing new therapies to prevent or ameliorate cardiovascular diseases.

According to the outcome of our previous studies, the rapid phenotypic transition of VSMCs induced by AngII has paralleled with the growing form of autophagy which is a process used for cellular renovation and is one of two major degradation system in eukaryotic cell example cancer [6,7]. And recently, Accumulating evidences suggests that autophagy is activated in VSMCs in vascular diseases and that it is important for plasticity of VSMCs, for example, it has been reported that autophagy stimulated proliferation of pulmonary artery smooth muscle cell [8,9]. While another researches indicated that Angiotensin II (Ang II) is one of important mediators of pathologic VSMC proliferation seen in several cardiovascular diseases. Given both AngII and autophagy have contributed to the phenotype modulation of VSMCs, our present study was designed to explore whether the AngII-induced transition from the contractile to the synthetic phenotype was triggered by an increase in autophagy and whether stimulation of autophagy was required for phenotype switching.

Methods

Cell culture

Human aortic SMCs (Lonza, Walkersville, MD) were cultured in growth media SmGM-2 (Lonza) in 5% Fetal Bovine Serum (FBS) at 37°C in a humidified 5% CO_2 incubator. All cells used in this study were between passages 5 and 7.VSMCs were serum-starved in starvation medium (0.5% FBS) for 24 hours and then subjected to stimulation with or without AngII at a final concentration of 1 μmol/L and monitored for 48 hours. In order to investigate the role of inhibitors of autophagy, VSMCs were serum-starved for 24 hours and then stimulated with AngII (1 μmol/L) for 48 hours. 3-MA (10 mM) or spautin-1 (10 μM) was added 30 min prior to AngII stimulation.

Western blot

Cell lyses were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Blots were blocked with 5% nonfat milk in phosphate-buffered saline (PBS) with 0.1% Tween20 and developed with diluted antibodies to Beclin1 (1:1000 dilution; Cell Signaling), LC3B (1:1000 dilution; Cell Signaling), SM22α (1:300 dilution; Santa Cruz Biotech), a-SMA (1:800 dilution; Sigma), Calponin (1:2000 dilution; Abcam), osteopontin (1:200 dilution; Sigma), AMPK (1:1000 dilution; Cell Signaling), Phospho-AMPK (Thr172) (1:1000 dilution; Cell Signaling), m-TOR (1:1000 dilution; Cell Signaling), Phospho-mTOR (Ser2481) (1:1000 dilution; Cell Signaling) and GAPDH (1:1000 dilution; Santa Cruz), followed by incubating with either IRDye 700 or 800 secondary antibodies and visualized using Odyssey Infrared Imaging System software (Li-Cor, Lincoln, NE).

mRNA isolation and real-time PCR

mRNA was isolated from human aortic VSMCs using the TRIzol®

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reagent (Invitrogen) and the concentration was determined by measuring absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific). A 20 µl reverse transcription reaction mixture containing 1 µg of mRNA, 10 units AMV (avianmyeloblastosis virus) reverse transcriptase, 0.4 µMpoly-T primer (dT18), 0.2 mM dNTP and 20 units RNasin® (Promega) was subjected to cDNA synthesis in a thermal cycler (Bio-Rad Laboratories). cDNA (2 µl) was then used for the amplification of the gene of interest by real-time PCR using SYBR Green (VWR).

Statistical analysis

All data were expressed as mean ± SEM. Data were analyzed for statistical significance by the unpaired Student t test or ANOVA. $P<0.05$ was considered statistically significant.

Results

Ang II induce VSMC phenotype switching

To investigate whether AngII modulates VSMC phenotype, we measured changes in molecular markers of contractile and synthetic VSMC phenotypes. Firstly, human VSMCs were deprived of serum for 24 h to induce cell-cycle arrest. The cells were then incubated with AngII (1 µmol/L) for 48 h. Using real-time PCR measured the relative mRNA of phenotypes molecular markers in AngII-treated VSMCs, and indicated that the contractile markers, such as a-SMA, SM22a, Calponin were decreased significantly; but the synthetic marker, such as Osteopontin (OPN) was obviously increased (Figure 1A). Relative protein abundance were measured by western blot. The level of a-SMA protein was decreased by 60% and OPN protein was increased approximate 4 fold (Figure 1B). Together, these findings suggest that AngII play an important role for VSMC phenotypic switch.

Ang II upregulate autophagy in VSMCs

Researchers have found that autophagy could manipulate the VSMC phenotype switch; We hypothesized that AngII was the upstream regulation factors of autophagy in VSMC [10,11]. To test the hypothesis, we assessed the effects of AngII on autophagy in VSMC and found that the VSMC autophagy was up regulated through examining LC3-II formation and Beclin1expression. Accordingly, in a time-dependent manner, the expression of LC3-II/ LC3-I and Beclin1, as determined by western blot, was markedly increased after exposure to AngII (Figure 2). In order to explore the signalling through which AngII activate autophagy in VSMC, we detected two major signaling that trigger autophagy, including mTOR and AMPK signaling pathway and found p-mTOR or p-AMPK has no statistical change (Figure 3). These results showed that AngII promotes autophagy via an mTOR and AMPK independent pathway.

Ang II induced autophagy is required for phenotype switching in VSMCs

To investigate whether autophagy plays a vital role in VSMC phenotype switching, we pre-treated VSMC with inhibitor of autophagy, 3-MA and spautin-1, prior to stimulating the cells with Ang II. It showed that spautin-1 decreased autophagy through examining LC3-II formation and Beclin1expression and inhibited transition from the contractile to the synthetic phenotype in AngII-treated VSMCs (Figure 4). Also, 3-MA had similar effect as spautin-1 on VSMC (Figure 5). Therefore, we confirmed that autophagy is required for phenotype switch in AngII-treated VSMCs.

Discussion

VSMCs phenotype switching has been shown to be a significant role on many cardiovascular diseases. There by understanding the

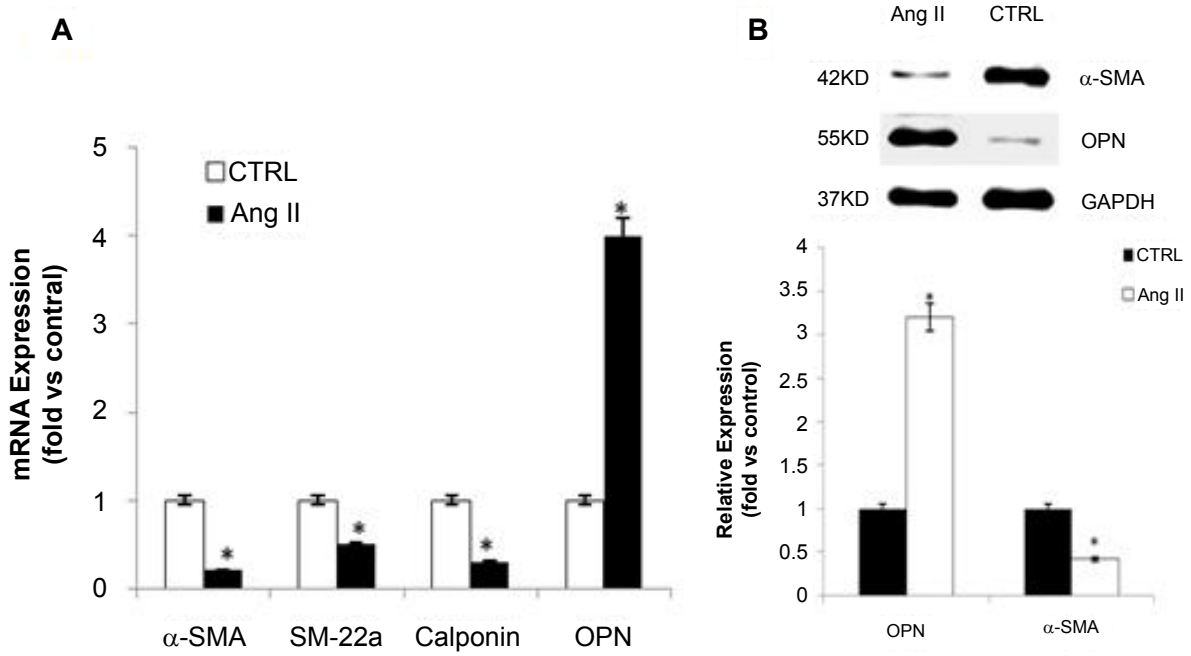

Figure 1: A) Human aortic smooth muscle cells cultured in AngII(1µmol/L) for 48h caused a significantly decreased expression of VSMC contractile marker genes, such as smooth muscle a-actin, smooth muscle 22a, Calponin, but increased expression of VSMC synthetic marker gene such as OPN ,as determined by quantitative reverse-transcription polymerase chain reaction (n=5). *P<0.05 compared with CTRL. **B)** The expression of contractile marker gene smooth muscle a-actin was decreased , but the expression of OPN was increased, after explored in AngII (1 µmol/L) for 48h, as determined by western blot(n=5), *P<0.05 compared with CTRL.

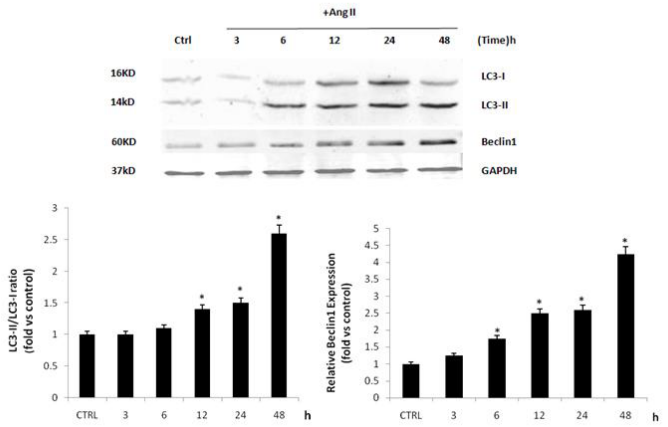

Figure 2: Human aortic smooth muscle cells were stimulated with AngII (1 μmol/L) for 0 to 48h, then the abundance of LC3-I, LC3-II and Beclin1were detected by western blot. Quantification of LC3-II/LC3-I and Beclin1 were tested (n=5). *P<0.05 compared with CTRL.

molecular mechanisms of the VSMC phenotype switching has become the focus of multiple studies [1,2]. The present research demonstrates a unique role of angiotensin II in manipulating VSMC phenotype through inducement of autophagy. We found that LC3-II formation, which is a validated marker of autophagy, has increased in the AngII-treated vascular smooth muscle cells, and we also noticed that Beclin-1 another important indicator of autophagy has risen as well [12]. Studies have identified that autophagy induced by PDGF that was essential for attaining the synthetic phenotype in VSMC and Beclin1 which was a mammalian orthologue of yeast Atg6, the first mammalian autophagy-related protein to be identified [10]. Beclin-1 plays an important role in regulating vacuolar sorting protein 34 (Vps-34, a class III phosphatidylinositol-3 kinase), and advances the formation of beclin-1-Vps34-Vps15 core complexes, which induce autophagy [13-15]. Our present study indicated that autophagy was also the downstream mediator of AngII signaling which induced VSMC phenotype switching.

Previous researchers have identified Ang II stimulated VSMC growth, hypertrophy and phenotype switching, increased collagen deposition and decreased dilation. The underlying molecular mechanisms of these pathological changes can be explained by Hilgers and Touyz as up regulation of many signaling pathways which comprise tyrosine kinases, mitogen activate-protein kinases, generation of reactive oxygen species,and activation of the small G protein RhoA and its target Rho kinase (ROCK) [16,17]. Furthermore, Pan found that both autophagy and the expression of the beclin-1 gene increased in AngII stimulated neonatal cardiomyocytes [18]. However, according to our study, the data demonstrated a novel picture in which upregulated autophagy by AngII plays significant role in modulating phenotypic switching of VSMCs.

Accordingly, in a time-dependent manner, the expression of LC3-II/ LC3-I and Beclin1 was dramtically increased after exposure to AngII. So those meant that autophagy was induced by AngII. Autophagy is a process used for cellular renovation and is one of two major degradation systems in eukaryotic cells which include proteasome and autophagy. Previous studies shown that proteasome actually promoted protein degradation, underlying transition from contractile to synthetic VSMC phenotype; comparatively less has been reported regarding autophagy's role in VSMC phenotype switching [19]. However, our present study suggested autophagy might be an essential regulator of VSMC phenotype through degrading contractile proteins during cellular transition to synthetic phenotype, the inhibitors of autophagy such as 3-MA and Spautin-1 stabilized the contractile phenotype. Although 3-MA inhibits autophagy via inhibiting the PI3K-Akt pathway, which is also important in regulating cell proliferation; Spautin-1 prevents autophagy by inhibiting the deubiquitinases ubiquitin-specific peptidase 10 (USP10) and ubiquitin-specific peptidase 13 (USP13), which leads to Beclin1 and Vps34 (vacuolar protein sorting-associated protein 34, which also known as PI3K III degradation) [20,21]. However, our data showed that neither 3-MA nor Spautin-1 had an apparent effect on the expression of the VSMC contractile proteins.

The induction of autophagy by AngII does not appear to be mediated by AMPK or mTOR. Previous studies have identified that AngII induced activation of JNK and NF-κB to promote the expression of MMP-2/9 and VSMC inflammation, proliferation and migration [22,23]. Recently, some researches showed that JNK phospholated Bcl-2 and Bcl-xL and dissociated of Bcl-2 or Bcl-xL from Beclin1 which induce the autophagy; NF-κB promoted autophagy by regulating Beclin1 expression [24,25]. So we supposed that AngII induced autophagy through JNK and/or NF-κB signaling pathway. But this is needed to be further researched. As previous studies shown autophagy

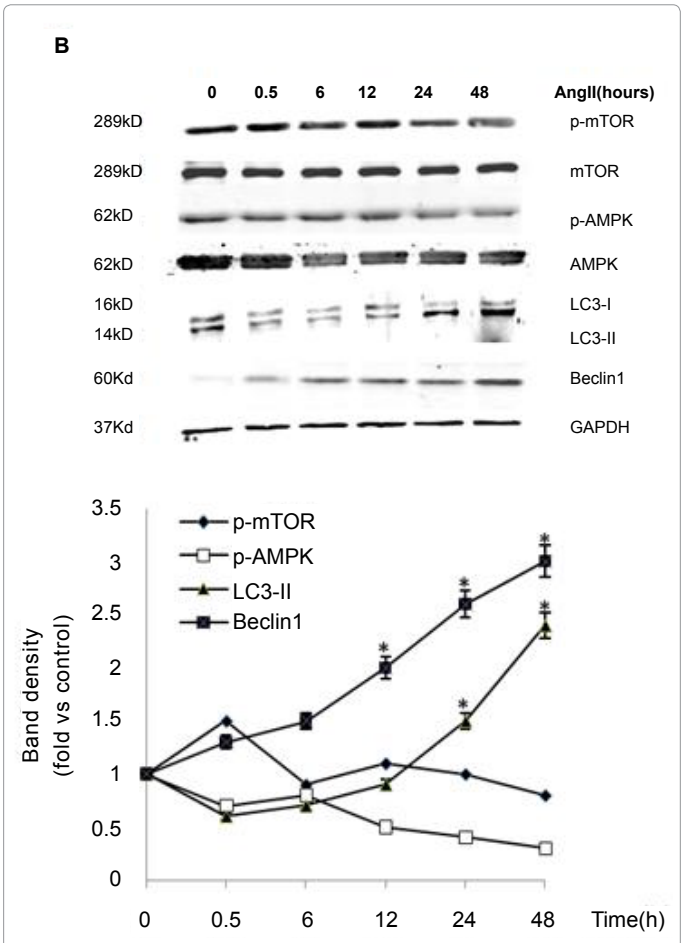

Figure 3: Immunoblot analysis of human aortic smooth muscle cells after AngII treatment. Western blot of mTOR, AMPK phosphorylation as well as LC3-I, LC3-II and Beclin 1. Each phosphorylated protein was normalized to its non- phosphorylated protein. Quantification of the changes were shown (n=5). *P<0.05 compared with zero.

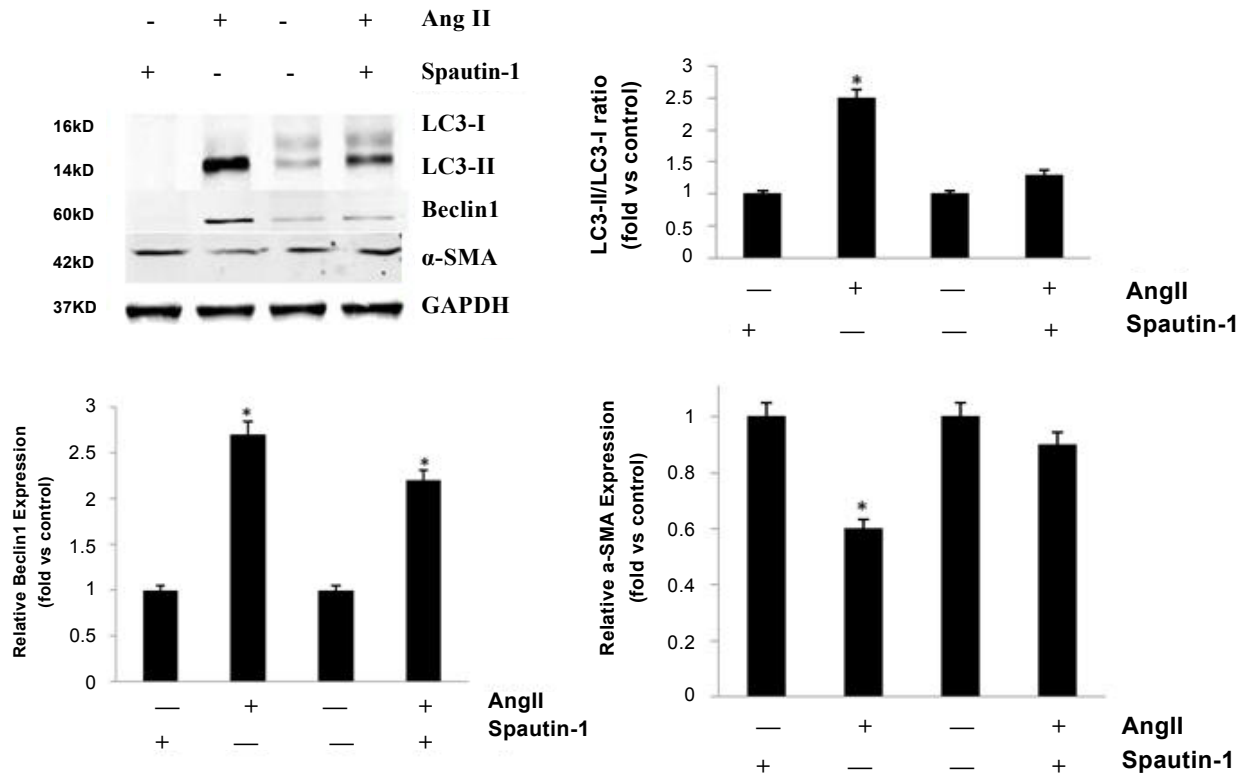

Figure 4: Pre-treated human aortic smooth muscle cells with inhibitor of autophagy spautin-1 (10 μM) prior to stimulating the cells with Ang II, then the expression of LC3-II,LC3-I,Beclin1 and contractile protein were detected by western blot. Quantification of LC3-II/LC3-I, Beclin1and smooth muscle a-actin were tested (n=5). *P<0.05 compared with untreated cells.

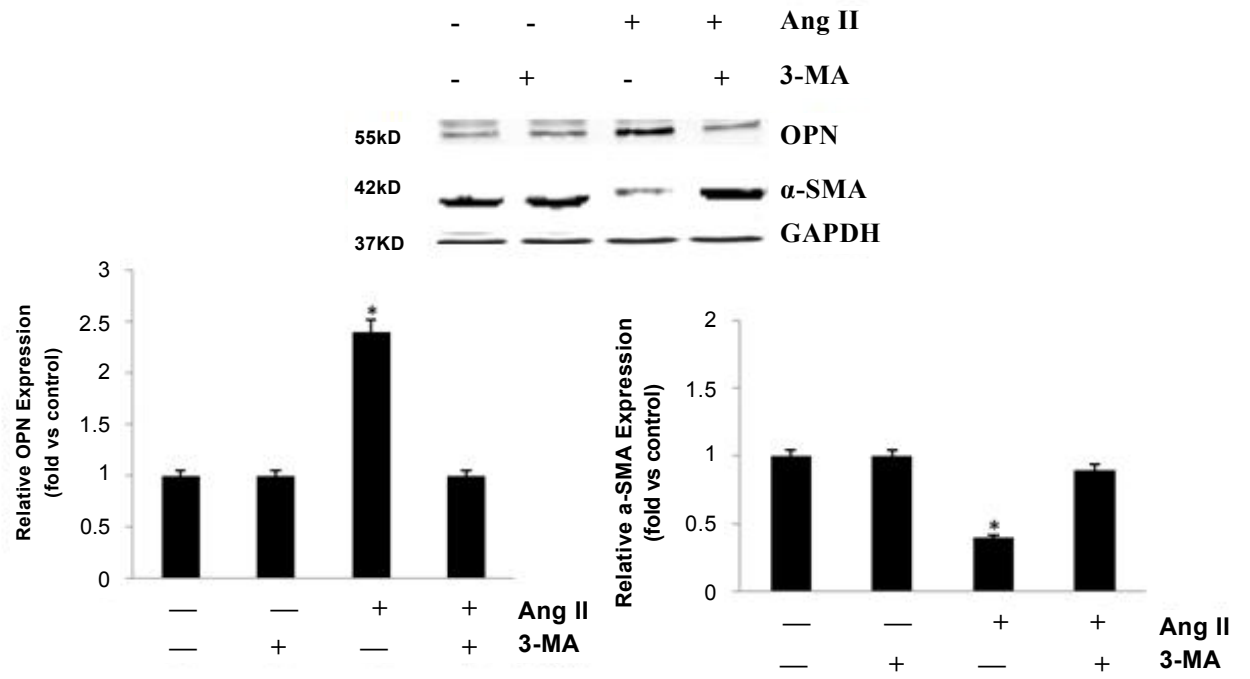

Figure 5: Pre-treated human aortic smooth muscle cells with inhibitor of autophagy 3-MA (10 mM) prior to stimulating the cells with Ang II, then the expression of the contractile and synthetic proteins were detected by western blot. Quantification of smooth muscle a-actin and OPN were tested (n=5). *P<0.05 compared with untreated cells.

could be activated by many factors related to the disease process in VSMC, such as reactive species derived from oxidative stress, ER stress, cytokines and growth factors, metabolic stress and so on [10,26-29]. Many of the autophagy-inducing stimuli promote conversion to synthetic phenotype in VSMC, but other inducers, such as rapamycin, prevent VSMC phenotype switching [30]. How the different form of autophagy could play a distinct role on VSMC phenotype switching remains to be elucidated.

In summary, the present study identifies a novel mechanism of VSMC phenotype conversion by which autophagy is activated by AngII. Although the mechanism of how autophagy modulate the VSMC phenotype still need to be further studied, a current hypothesis with respect to transition to the synthetic phenotype is that autophagy removes contractile elements, which integrates with known transcriptional programs to hasten phenotype transition. Further understanding of how autophagy regulates the function of VSMC will undoubtedly be helpful for therapy of many cardiovascular diseases.

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